Journal of Medical Virology 60:107–112 (2000)

Genotypic Differences in the Hepatitis B Virus Core Promoter and Precore Sequences During Seroconversion From HBeAg to Anti-HBe

Jonas Bläckberg and Karin Kidd-Ljunggren*
Department of Infectious Diseases, University Hospital, Lund, Sweden

Hepatitis B virus (HBV) strains from anti-HBe positive patients often show specific mutations in the precore gene, the core promoter region, or both. The dynamics of seroconversion in relation to the appearance of these mutations has not been studied and compared between defined HBV genotypes. Samples from patients followed during seroconversion from HBeAg to anti-HBe were amplified by polymerase chain reaction (PCR), sequenced and genotyped. Among 16 sets of samples, 6 belonged to genotype A, 6 to genotype D, 2 to genotype B, 1 to genotype C, and 1 to genotype E. Whereas strains from genotypes B, C and E showed changes in the core promoter, precore codon 28 or both, genotype A and D strains displayed a different pattern. In 4 of 6 anti-HBe positive samples from genotype A, the precore had a wild-type sequence while the core promoter sequence showed a specific TGA mutation. In another genotype A strain a precore stop mutation was preceded by a mutation in codon 15, thus conserving base-pairing at the pregenomic RNA level in this region. In contrast, all genotype D strains showed wild-type sequences in both the core promoter and precore codon 28 in pre- and post-seroconversion samples. Thus, in 8 patients with a mean follow-up time of 17 months, wild-type sequences in both the core promoter and precore codon 28 were found after seroconversion to anti-HBe. This study also confirmed, for genotype D, that HBeAg seroconversion often occurs earlier than genomic conversion. ***J. Med. Virol. 60:107–112, 2000.***

KEY WORDS: HBV; wild-type; mutation; temporal

INTRODUCTION

During infection with hepatitis B virus (HBV), a soluble protein is found in the serum of almost all acutely and a proportion of chronically infected patients. This protein, the HBeAg [Magnius and Espmark, 1972], was used for many years as the main infectivity marker of HBV [Shikata et al., 1977]. Seroconversion from HBeAg to anti-HBe was taken originally to signify a fall in viral replication and therefore of infectivity. It is still used in some studies as a marker of successful treatment with interferon [Wong et al., 1993].

As tests for the detection of HBV DNA became available more widely, it became clear that a proportion of anti-HBe positive patients carried replicative virus and were therefore infectious [Burk et al., 1994]. In a study by Carman et al. [1989], a mutation leading to a translational stop in precore codon 28 was seen to be associated with anti-HBe positivity in the infected patient. This mutation would prevent the production of HBeAg and has since been reported by numerous groups. It has been associated with more aggressive liver disease and with outbreaks of fulminant hepatitis [Omata et al., 1991; Liang et al., 1991].

Transcription of the precore and core genes is directed from the so-called core promoter region. A change from 1762 - 1764 AGG to TGA (numbering by Okamoto et al., [1988]) in the core promoter was first seen by Okamoto et al. (1994) to be associated with the HBeAg/anti-HBe status of the patient. In a cross-sectional study, both the core promoter mutations and the precore codon 28 mutation were found to be highly and equally significant for the HBe/anti-HBe phenotype displayed [Kidd-Ljunggren et al., 1997].

The dynamics of seroconversion in relation to the appearance of the precore codon 28 mutation has been studied by Lai et al. [1994]. A gradual takeover of mutated over wild-type strains seems to occur. In a recent

Grant sponsor: Crafoord Foundation; Grant sponsor: Bristol-Myers Squibb Research Foundation; Grant Sponsor: Swedish Society of Medicine; Grant sponsor: Royal Physiographic Society of Lund; Grant sponsor: Swedish Medical Research Council; Grant number K98-16X-11592-03A.

*Correspondence to: Karin Kidd-Ljunggren, M.D., Ph.D., Department of Infectious Diseases, University Hospital, S-221 85 Lund, Sweden. E-mail: Karin.Kidd@infek.lu.se

Accepted 16 July 1999

study by Chan et al. [1999], Chinese patients from Hong Kong, divided into two groups by a variation in precore codon 15, were followed through seroconversion. Only 2 of 26 patients had wild-type sequences in the core promoter and precore codon 28 after seroconversion.

A study was undertaken to determine whether the pattern that appeared in the core promoter and precore codon 28 during seroconversion from HBeAg to anti-HBe would vary between the different HBV genotypes. In particular, the sequence pattern of well-defined genotype A strains was examined. Base-pairing at the pregenomic RNA level in the precore region, which is essential for replication [Junker-Niepmann et al., 1990], precludes the development of the precore stop codon in genotype A strains [Li et al., 1993]. These strains would therefore have to use other mechanisms to prevent HBeAg formation.

MATERIALS AND METHODS

Serum Samples and HBV Marker Tests

Samples from 51 HBV-infected patients who had been followed with routine serological testing during seroconversion from HBeAg to anti-HBe were examined retrospectively. The samples had been stored at −20°C. None of the patients had been treated with interferon or specific antiviral drugs. From each of the 51 patients at least two samples were analyzed by polymerase chain reaction (PCR), namely, an HBeAg positive and anti-HBe negative sample, and an HBeAg negative and anti-HBe positive sample. If there were more than two samples available, the last HBeAg positive and the first anti-HBe positive samples were chosen for the initial PCR. In case no. 6 the first sample was positive for both markers. Commercial radioimmunoassays (Abbott Laboratories, North Chicago, IL) were used for the detection of HBsAg, HBeAg and anti-HBe.

Enzymatic Amplification and Sequencing

DNA was extracted from serum and amplified by PCR as described [Ljunggren and Kidd, 1991], using slightly modified cycling temperatures. Oligonucleotide primers KL28 (5′GAG ACC ACC GTG AAC GCC 3′) and KL6 (5′GGA AAG AAG TCA GAA GGC A 3′) were used to amplify the core promoter and precore regions. Ten to 100 genome copies can be detected using this protocol. The pre-S and S genes were amplified using KL12 (5′GGG TCA CCA TAT TCT TGG G 3′) and KL33 (5′ACC ACT GAA CAA ATG GCA CTA G 3′). The PCR primers were then used for direct sequencing of the amplified DNA by the method of Kretz et al. [1989]. Standard precautions were taken to prevent carry-over of DNA [Kwok, 1990; Ljunggren and Kidd, 1991] and there was no evidence of contamination.

Determination of Genotypes

Sequences obtained by direct sequencing were aligned with strains which genotypically were well defined. The genotype of each strain was determined by comparing key nucleotide positions in the core promoter, precore and S genes [Norder et al., 1993; Kidd-Ljunggren et al., 1995a,1997; Kidd and Kidd-Ljunggren, 1996].

RESULTS

In the samples from HBV-infected patients who had been followed through seroconversion from HBeAg to anti-HBe, HBV DNA was detected by PCR in the first (HBeAg positive) sample from all 51 cases. In 35 cases, the second, HBeAg negative sample was negative by PCR, thus precluding further analysis. For the remaining 16 cases, an attempt was made to amplify all other available samples. In total, 52 samples were tested (Table I). There was a mean time of 26 months between the first and the last sample (range 1.5–79 months) and a mean time of 12 months between the last HBeAg positive and the first HBeAg negative sample (range 1.5–28 months).

The sequences obtained for each sample, in relation to the HBeAg/anti-HBe phenotype displayed in the sample, are presented in Table I. The core promoter sequence $A^{1762}GG^{1764}$ was considered to be the wild-type, whereas a mutated core promoter sequence was $T^{1762}GA^{1764}$ (Fig. 1). The presence of A^{1896} instead of G indicates a precore codon 28 stop mutation (Fig. 2). Six sets of samples belonged to genotype A, 6 to genotype D, 2 to genotype B and 1 each to genotypes C and E. Four of the genotype D strains showed homology to the genotype D strains seen in Swedish drug addicts (data not shown).

The sequence patterns seen during seroconversion, particularly at position 1762, 1764, and 1896, differed considerably amongst the 16 cases (Table I). In the HBeAg positive samples, 3 of 16 strains (patients 2, 8 and 9) had a stop mutation in precore codon 28. In one of these cases (patient 9) there was a mixture of wild type and mutated strains. The stop mutation only appeared in one additional sample (patient 7) after seroconversion to anti-HBe. Thus, in 12 of 16 cases, the patient had a wild-type precore sequence after seroconversion to anti-HBe. In 4 of these 12 cases (patients 3, 5, 15, and 16) there were multiple anti-HBe-positive samples available, all displaying the wild-type precore sequence. The mean anti-HBe positive follow-up time for these four patients was 10 months (range 2½–24 months). In one patient (no. 2) the genotype A strain developed the precore stop codon in the last available HBeAg positive sample. This was preceded by a change from C at position 1858 in the first sample, to T in the second sample. The remaining anti-HBe positive samples had T 1858 (Fig. 3).

In the core promoter region, the sequence patterns were similar between the first HBeAg-positive sample (10 of 16 samples had the wild-type sequence) and the last anti-HBe positive sample (9 of 16 with wild-type). During the transition from HBeAg-positive to anti-HBe positive samples, patient no. 7 had an intermediate sample with mixed wild-type and mutated core promoter sequence. In 3 of the 4 patients who developed

TABLE I. Mutations in the Core Promoter and Precore Region Found in Serial Samples From Patients During Seroconversion From HBeAg to Anti-HBe

Pat.	Age/sex	Genot.	HBeAg/ a-HBe	C.p.[a]	Pre-c28[b]
1	49/M	A	+/–	mix	wild
			–/+	TGA	wild
2	46/M	A	+/–	TGA	wild
			+/–	TGA	wild
			+/–	TGA	stop[c]
			–/–	TGA	stop
			–/+	TGA	mix
3	10/M	A	+/–	wild	wild
			–/+	wild	wild
			–/+	wild	wild
4	34/M	A	+/–	TGA	wild
			+/–	TGA	wild
			+/–	TGA	wild
			–/+	TGA	wild
5	22/M	A	+/–	TGA	wild
			–/+	TGA	wild
			–/+	TGA	wild
			–/+	TGA	wild
6	9/F	A	+/+	wild	wild
			–/+	wild	wild
7	31/M	B	+/–	wild	wild
			–/+	mix	wild
			–/+	TGA	wild
			–/+	TGA	stop
			–/+	TGA	stop
8	18/F	B	+/–	wild	stop
			–/+	wild	stop
9	11/F	C	+/–	TGA	wild
			+/–	TGA	wild
			+/–	TGA	mix
			–/+	TGA	stop
			–/+	TGA	stop
10	30/F	D	+/–	wild	wild
			–/+	wild	wild
11	20/M	D	+/–	wild	wild
			–/+	wild	wild
12	30/M	D	+/–	wild	wild
			–/+	wild	wild
13	8/M	D	+/–	wild	wild
			+/–	wild	wild
			+/–	wild	wild
			–/+	wild	wild
14	2/F	D	+/–	wild	wild
			+/–	wild	wild
			–/+	wild	wild
15	17/M	D	+/–	wild	wild
			–/+	wild	wild
			–/+	wild	wild
16	12/F	E	+/–	TGA	wild
			–/+	TGA	wild
			–/+	TGA	wild
			–/+	TGA	wild

[a]Core promoter wild-type sequence is $A^{1762}GG$. Mixed sequence designation here means that both A and T are present at position 1762.
[b]The precore codon 28 wild-type sequence is $TG^{1896}G$, whereas an A at position 1896 leads to a stop. Mixed sequence designation here means that both G and A are present at position 1896.
[c]In this strain the development of a stop in precore codon 28 at position 1896 was preceded by an upstream change from C to T at position 1858 in the previous sample.

stop codons at position 1896, core promoter mutations preceded the precore mutations (patients 2, 7, and 9).

There were in total 8 patients who, in their last available anti-HBe positive sample, had neither specific mutations in the core promoter nor a translational stop in precore codon 28 to explain the lack of detectable HBeAg. The mean follow-up time for these patients was 17 months. The core promoter and precore sequences from these samples were examined in more detail. No other sequence variations preventing HBeAg production could be found. One patient (no. 3) had a consistent, conservative mutation in the virus genome throughout seroconversion, from 1888 G to A. This mutation serves to increase the stability of the upper stem of the encapsidation signal at the pregenomic RNA level [Junker-Niepmann et al., 1990].

Fig. 1. Sequencing gels of the core promoter region. Order of lanes is GCAT. The $A^{1762}GG$ and $T^{1762}GA$ sequences are shown by asterisks. **A:** wild-type (AGG) strain. **B:** mutated (TGA) strain.

The sequence and marker combinations differed considerably between genotypes. Whereas mutations in the core promoter often preceded seroconversion from HBeAg to anti-HBe in genotype A strains, no core promoter changes were seen in strains belonging to genotype D. All four strains belonging to genotypes B, C and E had changes in the core promoter and/or precore codon 28, whereas all genotype D strains and two of six genotype A strains displayed wild type sequences throughout the follow-up. In patients no. 2, 4, and 9, the core promoter showed the TGA variation long before the actual seroconversion from HBeAg to anti-HBe occurred (25, 24, and 61 months respectively).

DISCUSSION

Despite its use for routine HBV serology for more than 20 years, the function of the HBeAg remains enig-

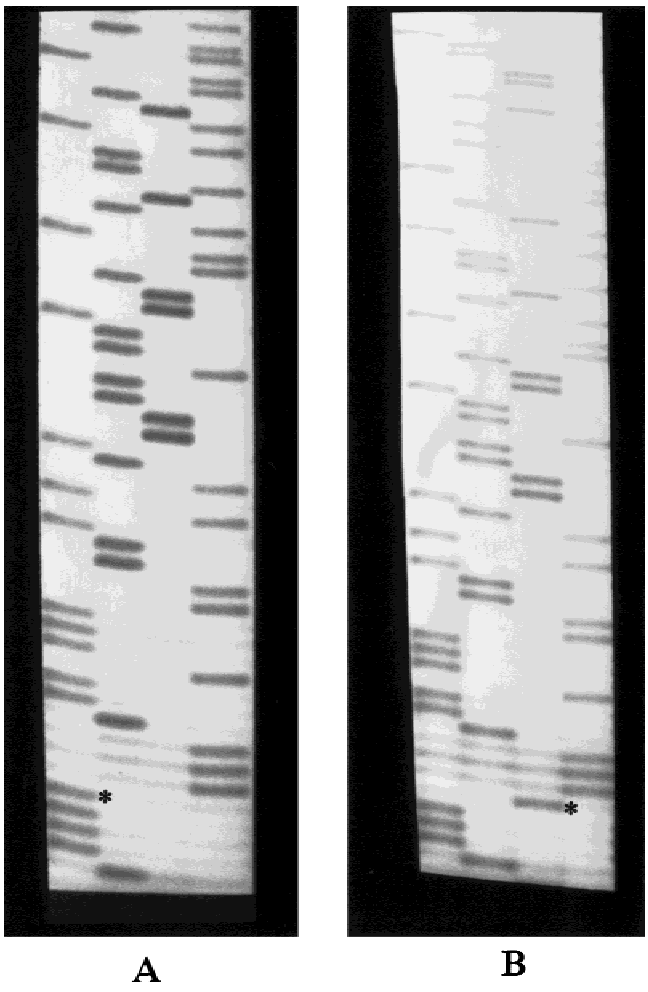

Fig. 2. Sequencing gels of the precore/core junction. Position 1896 is depicted by an asterisk. Order of lanes is GCAT. **A:** wild-type strain with G at position 1896. **B:** mutated strain with A at position 1896, leading to a translational stop.

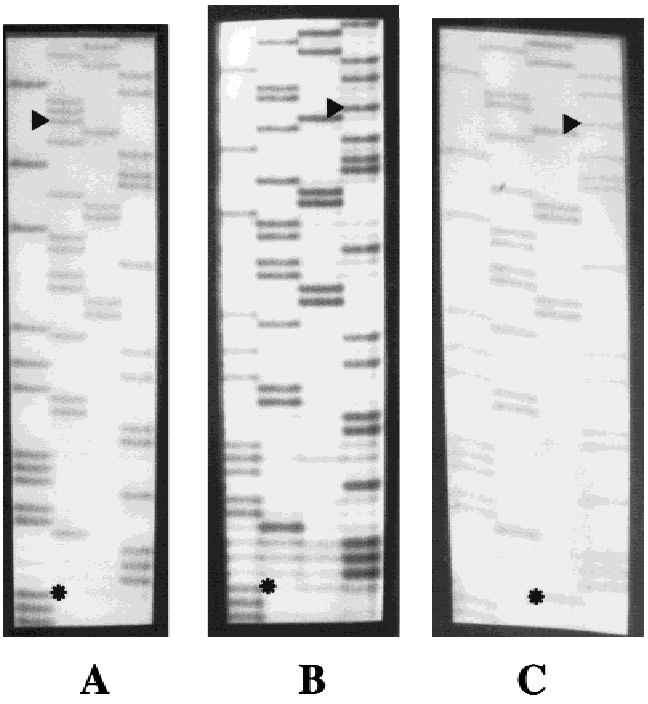

Fig. 3. Sequencing gels of the precore region from the genotype A strain infecting patient no. 2. Order of lanes is GCAT. Position 1858 is depicted by an arrow. Position 1896 is depicted by an asterisk. **A:** HBeAg positive and anti-HBe negative sample with C at position 1858 and G at position 1896. **B:** HBeAg positive and anti-HBe negative sample with T at position 1858 and G at position 1896. **C:** HBeAg negative and anti-HBe negative sample with T at position 1858 and A at position 1896.

matic and its role as a target for the immune system has yet to be confirmed. In the many reports where precore codon 28 sequences have been published and analyzed in the context of HBeAg seroconversion, exceptions to the rule of precore codon 28 stop mutation leading to anti-HBe seroconversion have been found [Okamoto et al., 1994; Kidd-Ljunggren et al., 1995a; Kidd-Ljunggren et al., 1997]. In some of these cases, mutations in the upstream core promoter, affecting the transcriptional regulation of the precore gene and thus of its protein expression, would explain why a precore wild-type strain may elicit an HBeAg negative phenotype [Okamoto et al., 1994; Kidd-Ljunggren et al., 1997; Inoue et al., 1998]. Most authors have found that the $T^{1762}GA^{1764}$ mutations lead to a reduced transcription of precore RNA, whereas there is still conflicting evidence about the effect on replication. Some authors have found enhanced replication in vitro with these mutations [Buckwold et al., 1996; Scaglioni et al., 1997; Moriyama et al., 1996], while Gunther et al. [1998] reported wild-type levels. The influence on HBe seroconversion of core promoter or precore mutations, or both, has therefore been well documented. It must be emphasized, however, that while these mutations can inhibit HBeAg synthesis, they may not have any immediate bearing on the production of anti-HBe.

In a recent study, Nishizono et al. [1997] studied sequential samples from three anti-HBe positive patients suffering from exacerbation of acute hepatitis. They found that the core promoter and precore stop mutations developed after exacerbation. In a large-scale study, seroconversion from HBeAg to anti-HBe was found to be a separate and earlier event in most patients than the corresponding genomic conversion from precore codon 28 wild-type to stop [Maruyama et al., 1998]. The temporal relationship between the appearance of specific mutation(s) and seroconversion from HBe to anti-HBe remains to be analyzed for the different genotypes.

After the first characterization of HBV strains into four different genotypes [Okamoto et al., 1988], this classification has largely replaced the earlier serological subtype system. One of the differences between genotypes is the lack of a precore codon 28 stop mutation in genotype A strains when anti-HBe develops. A likely explanation for this appears to be the structure of the precore region at the pregenomic level. During genomic replication there is a step of reverse transcription, which is initiated by a primer dislocation from the

stable pregenomic RNA stem-loop structure of the precore region to the upstream DR1. In genotype A strains, a C at position 1858 leads to disruption of the base-pairing in the lower stem of this stem-loop structure when the stop mutation (G to A) appears [Li et al., 1993; Kidd and Kidd-Ljunggren, 1996]. The levels of replication seem to decrease dramatically when this disruption occurs, and in most cases, patients infected with genotype A strains become PCR negative when they seroconvert to anti-HBe.

There seems to be a mechanism for the genotype A strains to cope with the precore codon 28 mutation. As seen in this study, case no. 2 was HBeAg positive and anti-HBe negative in the first sample, having a wild-type precore sequence (Fig. 3, Table I). Before the precore stop mutation (G to A) developed, the corresponding base on the opposite side of the stem (codon 15) changed from a genotype A-specific C to T, thus ensuring a stable base-pairing in this region. A similar observation was made by Li et al. [1993]. The precore stop mutation probably happens as frequently in genotype A strains as in the other genotypes. The only viable genotype A strains though, will be those that also manage to develop a corresponding mutation in precore codon 15, preventing disruption of the stem-loop structure.

In this study, the most marked difference in sequence patterns after seroconversion was not, as reported in a very recent study [Chan et al., 1999], between strains with C 1858 and T 1858, but between genotypes. All genotype D (T 1858) strains had wild type sequences in both the core promoter and precore codon 28, compared to two of ten strains from genotypes A (C 1858), B (T1858), C (T 1858) and E (T 1858). The comparatively high number of anti-HBe positive wild-type strains in this study may be due in part to the follow-up time. The average observation time in the study by Maruyama et al. [1998] was two to three years, compared to 17 months for the eight wild-type cases in the present study. It is therefore possible that several of the wild-type genotype D strains from anti-HBe positive patients in our study would have changed to precore stop mutants with a longer time of observation.

Another difference between the patients in this study and those in the report by Chan et al. [1999] is that more than half of their patients had been treated with interferon, whereas none of our patients had received interferon or any other antiviral treatment. Mutations in precore codon 28 and 29 have been seen to appear more often during seroconversion to anti-HBe in patients treated with interferon [Gunther et al., 1992].

This study shows that the pattern of core promoter and precore sequence mutations differs between HBV genotypes during seroconversion from HBeAg to anti-HBe. We also confirmed the observation, for genotype D (and some A) strains, that seroconversion is a temporally different, and often earlier event than genomic conversion in the precore [Maruyama et al, 1998] and core promoter regions. This lends support to the view that these mutations may appear as an escape mechanism after the immunological T-cell directed clearance of HBeAg-bearing hepatocytes.

ACKNOWLEDGMENTS

We thank Alistair Kidd for valuable discussions and Susanne Bengtsson for excellent technical help.

REFERENCES

Buckwold VE, Xu Z, Chen M, Yen TSB, Ou JH.1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 70:5845–5851.

Burk RD, Hwang LY, Ho GYF, Shafritz DA, Beasley RP.1994. Outcome of perinatal hepatitis B virus exposure is dependent on maternal virus load. J Infect Dis 170:1418–1423.

Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC.1989. Mutations preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 2:588–591.

Chan HLY, Hussain M, Lok ASF.1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 29:976–984.

Gunther S, Meisel H, Reip A, Miska S, Kruger DH, Will H.1992. Frequent and rapid emergence of mutated pre-C sequences in HBV from e-antigen positive carriers who seroconvert to anti-HBe during interferon treatment. Virology 187:271–279.

Gunther S, Piwon N, Will H.1998. Wild type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653)-T, A(1762)-T, and G(1764)-A mutations in the core promoter. J Gen Virol 79:375–380.

Inoue K, Yoshiba M, Sekiyama K, Okamoto H, Mayumi M.1998. Clinical and molecular virological differences between fulminant hepatic failures following acute and chronic infection with hepatitis B virus. J Med Virol 55:35–41.

Junker-Niepmann M, Bartenschlager R, Schaller H. 1990. A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J 9:3389–3396.

Kidd AH, Kidd-Ljunggren K.1996. A revised secondary structure model for the 3′-end of hepatitis B virus pregenomic RNA. Nucleic Acids Res 24:3295–3301.

Kidd-Ljunggren K, Öberg M, Kidd AH.1995a. The hepatitis B virus X gene: analysis of functional domain variation and gene phylogeny using multiple sequences. J Gen Virol 78:1469–1478.

Kidd-Ljunggren K, Ekdahl K, Öberg M, Kurathong S, Lolekha S.1995b. Hepatitis B virus strains in Thailand: genomic variants in chronic carriers. J Med Virol 47:454–461.

Kidd-Ljunggren K, Öberg M, Kidd AH.1997. Hepatitis B virus X gene 1751 to 1764 mutations: implications for HBeAg status and disease. J Gen Virol 78:1469–1478.

Kretz KA, Carson GS, O'Brien JS. 1989. Direct sequencing from low-melt agarose with Sequenase. Nucleic Acids Res 17:5864.

Kwok S.1990. Procedures to minimize PCR-product carry-over. In: Innis MA. editor. PCR protocols. A guide to methods and applications. San Diego:Academic Press Inc. p 142–145.

Lai ME, Solinas A, Mazzoleni AP, Deplano A, Farci P, Lisci V, Porru A, Tocco A, Balestrieri A. 1994. The role of pre-core hepatitis B virus mutants on the long-term outcome of chronic hepatitis B virus hepatitis. J Hepatol 20:773–781.

Li J-S, Tong S-P, Wen Y-M, Vitvitski L, Zhang Q, Trepo C.1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. J Virol 67:5402–5410.

Liang TJ, Hasegawa K, Rimon S, Wands JR, Ben-Porath E.1991. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. New Engl J Med 324:1705–1709.

Ljunggren K, Kidd AH.1991. Enzymatic amplification and sequence analysis of precore/core DNA in HBsAg-positive patients. J Med Virol 34:179–183.

Magnius LO, Espmark LÃ.1972. New specificities in Australia

antigen positive sera distinct from Le Bouvier determinants. J Immunol 109:1017–1021.

Maruyama T, Kuwata S, Koike K, Iino S, Yasuda K, Yotsuyanagi H, Moriya K, Maekawa H, Yamada H, Shibata Y, Milich DR. 1998. Precore wild-type DNA and immune complexes persist in chronic hepatitis B after seroconversion: no association between genome conversion and seroconversion. Hepatology 27:245–253.

Moriyama K, Okamoto H, Tsuda F, Mayumi M. 1996. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. Virology 226:269–280.

Nishizono A, Kohno K, Takita-Sonoda Y, Hiraga M, Terao H, Fujioka T, Nasu M, Mifune K. 1997. Sequential analyses of the mutations in the core upstream and precore regions of hepatitis B virus genome in anti-HBe positive-carriers developing acute exacerbation. J Med Virol 53:266–272.

Norder H, Hammas B, Lee S-H, Bile K, Courouce A-M, Mushawar IK, Magnius LO.1993. Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. J Gen Virol 74:1341–1348.

Okamoto H, Tsuda F, Sakugawa H, Sastroewigjno RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e-antigen. J Virol 68:8102–8110.

Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. 1991. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. New Engl J Med 324:1699–1704.

Scaglioni PP, Melegari M, Wands JR.1997. Biological properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. Virology 233:374–381.

Shikata T, Karasawa T, Abe K, Uzava T, Suzuki H, Oda T, Imai M, Mayumi M, Moritsugu Y. 1977. Hepatitis B e antigen and infectivity of hepatitis B virus. J Infect Dis 136:571–576.

Wong DKH, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J.1993. Effect of alpha-interferon treatments in patients with hepatitis B e antigen-positive chronic hepatitis B. A metaanalysis. Ann Intern Med 119:312–323.